Appendix

Protein Coated Lactose Crystals

The objective of this experiment was to investigate any changes in crystallisation behaviour when the sugar crystals are co-precipitated with a protein using the coprecipitation process and to determine how these effects are influenced when the percentage loading of the protein is changed.

For each of the anti-solvents used in the solvent screen two precipitations were carried out, one with a low loading (approx. 5%) and one with a high (approx. 30%) loading of protein. The protein used for the experiments was catalase. Immediately prior to the crystallisation enough catalase to achieve the appropriate percentage loading was dissolved in 0.25ml aqueous solution. The crystallisations were carried out using the batch process.

SEM images of the sample were analysed for lactose where the sugars had been crystallised using ethanol as the anti-solvent. For lactose with both a low and a high loading of catalase the size of the agglomerates remained the same. However, for both loadings of catalase the size of the individual particles making up the agglomerates were smaller. Each agglomerate was also formed from a larger number of needles making the agglomerates more spherical. The needles in the sample with the high loading of catalase are joined together with catalase, making the agglomerates look like a solid sphere rather than a ball of needles.

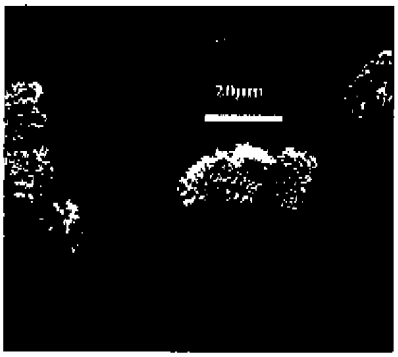


Figure: SEM of Lactose particles with 5% loading of catalase.

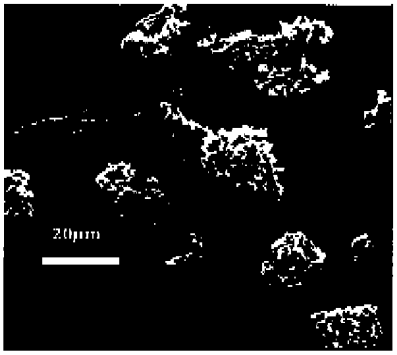


Figure: SEM of lactose particles with 30% loading of catalase.

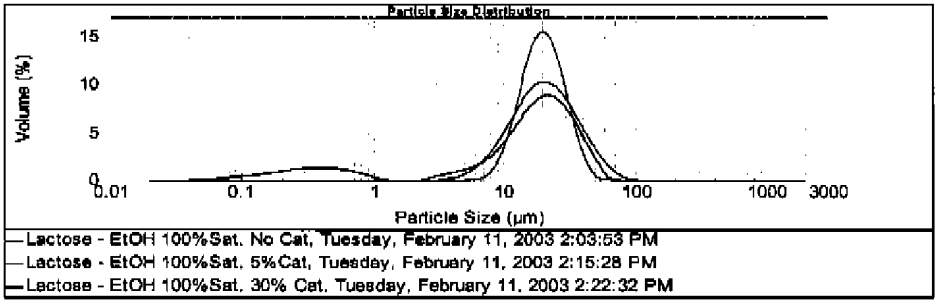


Figure 1: Size distribution of lactose with proposed loadings of catalase : 0% (red line), 5% (green line) and 30% (blue line).

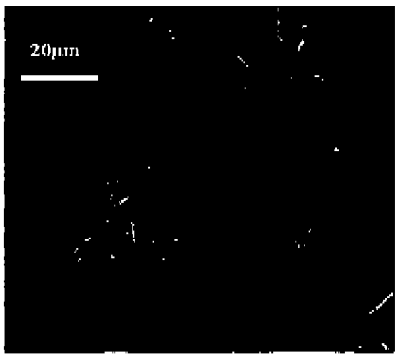
Continuous Flow System vs Batch

To investigate the differences between the batch and continuous flow processes an experiment was carried out to compare the powders produced from the two processes. From the SEM images and sizing data obtained from the samples formulated from the batch process three anti-solvents were chosen to use on the continuous flow system, acetone, ethanol, and propan-2-ol were chosen for lactose. The crystallisations were all carried out using the continuous flow system. The samples were analysed by optical microscopy, scanning electron microscopy and laser diffraction. The concentration of catalase was analysed using UV spectroscopy.

Lactose

Acetone

Catalase coated microcrystals were produced but with slightly different morphology from those seen from the batch process. The sample was a mixture of plate like and irregular shaped crystals that were approximately 20µm by 2µm in size. Any agglomerates were easily dispersed and the size distribution in the sample was small.



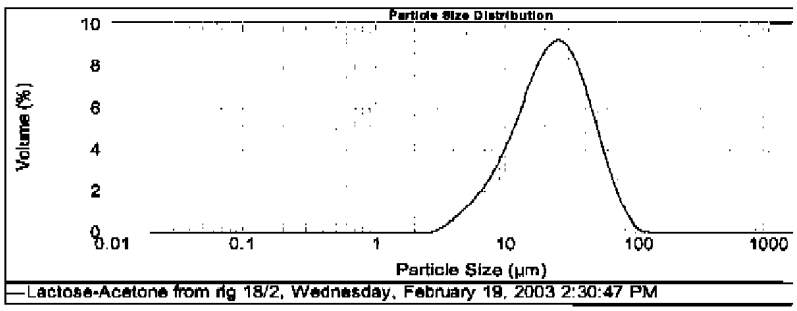


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Figure: SEM image of lactose particles, crystallised using aceone

Figure 2: Size distribution of lactose when acetone used as anti-solvent. Formulated on continuous flow system, measured after 30 seconds sonication.

Ethanol

The morphology of the sample produced using the continuous flow rig was variable. The first run produced large cube like structures that were approximately 10μm by 60μm. The sample was then dissolved back up in the aqueous solution and the experiment repeated. This time the crystal morphology was the same as that of the batch process. The agglomerates were smaller and the size distribution much smaller.

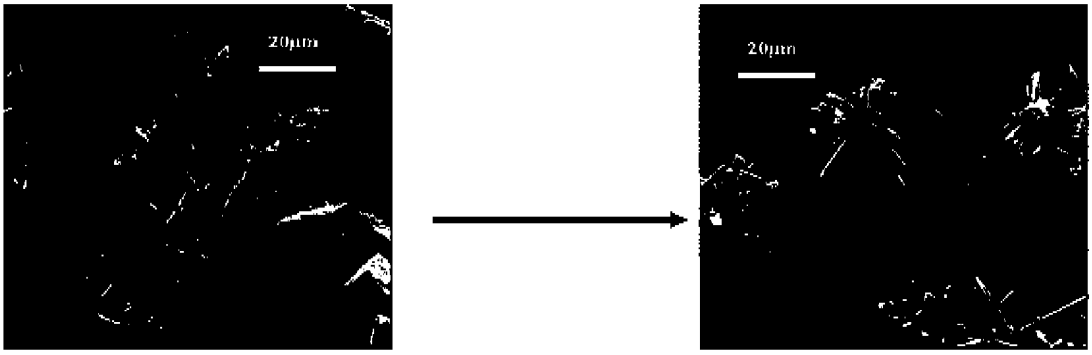

Figure 3: SEM images of lactose particles when ethanol used as anti-solvent.

Figure: SEM images of repeat

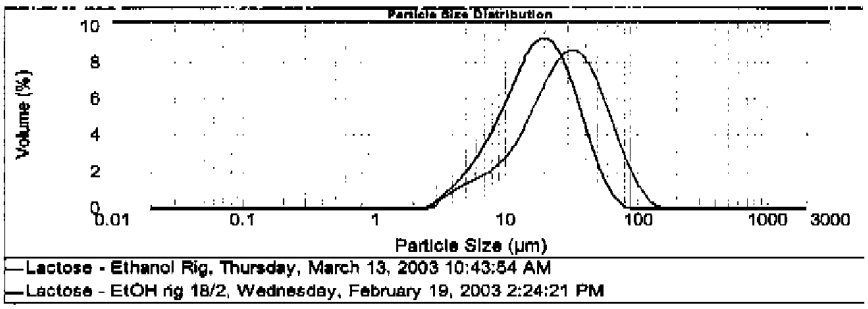

Figure: Size distributions from lactose samples (1) and (2). Crsytallised using ethanol as anti-solvent. Measured after 30 seconds sonication.

Propan-2-ol

The morphology of the crystals was similar to that produced form the batch system. The size of

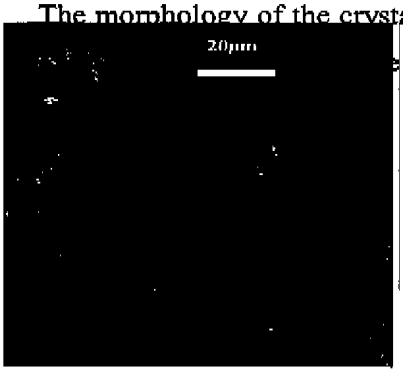

Figure: SEM image of lactose particles when propan-2-ol used as anti-solvent.

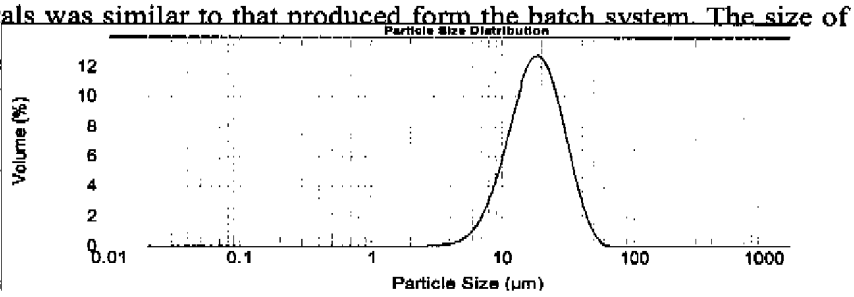

Figure: Size distribution of lactose when propan-2ol used as anti-solvent. Formulated on continuous flow system. Measured after 30 seconds sonication.

Catalase Loading

In order for this to be a successful technique the process has to retain the protein. A summary of the protein loadings obtained for batch and continuous is shown below.

Table 1: Comparison of the proposed and the actual loadings of

Sugar	Anti-Solvent	Process	Proposed Loading	Actual Loading
Lactose	Acetone	Batch	5%	3%
Lactose	Ethanol	Batch	30%	22%
Lactose	Propan-2-ol	Batch	6%	4%
Lactose	Acetone	Continuous	4%	3%
Lactose	Ethanol	Continuous	4%	2%
Lactose	Propan-2-ol	Continuous	4%	2%

Catalase following continuous and batch precipiation,

There are no major variations in the proposed and actual loadings of catalase between PCMC prepared by the batch or continuous methods suggesting the methods are comparable in efficiency.

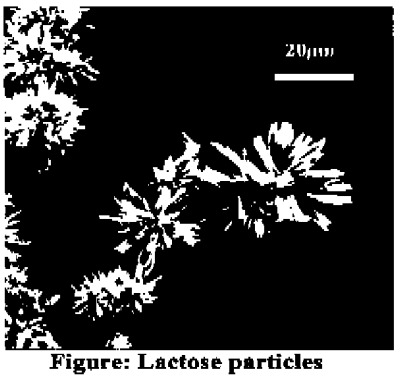

Figure: Lactose particles without catalase.

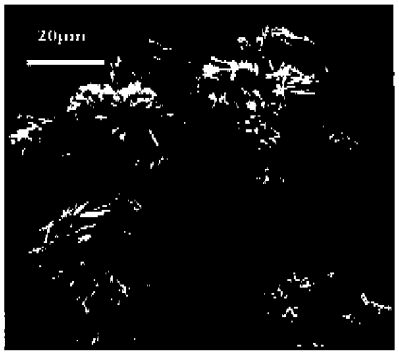

Figure 4: Lactose particles with catalse.

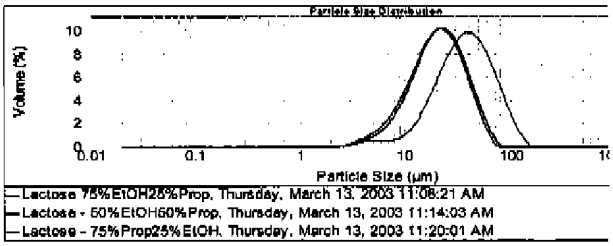

Figure: Size distribution for ethanol/propan-2-ol system. Without catalase.

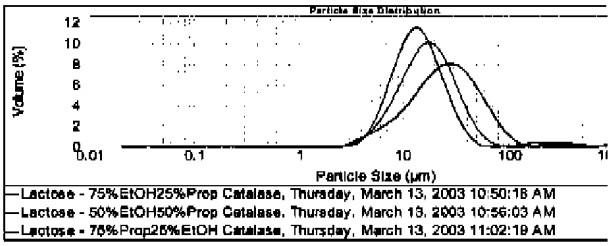

Figure: Size distribution for ethanol/propan-2-ol system. With catalase.

Batch Procedure

Each precipitation was carried out in a 25ml glass vial containing a magnetic flea. 4.75ml of saturated anti-solvent was added and the magnetic stirrer plate set for 500rpm. 0.25ml of the saturated aqueous solution was then added drop-wise from a syringe, the rate of which was kept constant by adding 1 drop every 10 seconds. These volumes were chosen so that the final water content in solution was 5% v/v. The same syringe was used for each experiment in order to keep the drop size constant. After all the aqueous solution had been added the resulting suspension was removed from the stirrer plate after 60 seconds and filtered immediately. The dried crystals were then weighed and stored as a dry powder.

Continuous Flow Process

Using a flow cell and two HPLC pumps a continuous flow system was constructed to enable larger scale production of the micro-crystals and PCMCs and to show if there were any differences in crystallisation habit seen between the batch and continuous processes. A schematic of the continuous flow rig is shown below.

The two pumps, one for the anti-solvent and one for the aqueous solution, were connected to a flow cell that mixes the two solutions together. The mixed solution is then forced up through the top of the flow cell through a length of tubing to a collection vessel.

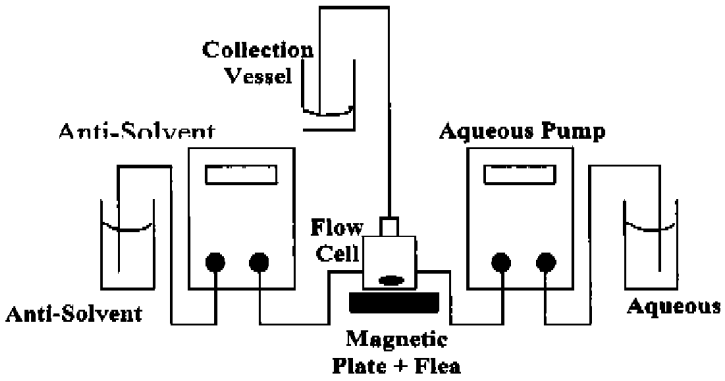

Figure: Schematic of the continuous flow process.

Continuous Procedure

Before each experiment the rig was flushed with anti-solvent to ensure that that there was negligible water remaining in the system. The flow-rate of the anti-solvent pump was put at 4.75ml/min and the aqueous pump at 0.25 ml/min, this meant that the resulting suspension that was collected contained 5% H_2O v/v, the same as the batch process. The pumped solution was collected until precipitating crystals could be seen in the collection vessel, the solution was collected for a further two minutes before replacing the container with clean one and discarding the initial solution collected in the first container. The system was then left to run for 15 minutes, the resulting suspension was filtered, dried and weighed.

Examples of the use of other coprecipitants to produce protein coated micro-cystals (PCMC)

Aim: Coprecipitation of trypsin on potassium sulfate (K_2SO_4) PCMC from propan-2-ol (IPA) using continuous flow coprecipitation systems.

Experimental: Tryspin (Bovine Pancreatic) Cat. No. T1426 and potassium sulfate Cat. No. 121K0043 and IPA were supplied by Sigma Aldrich UK.

An aqueous solution containing 2.56 mg/ml trypsin and 100 mg/ml potassium sulfate was prepared. This solution was precipitated into IPA saturated with potassium sulfate via the two line system, whereby the aqueous solution was delivered at 4 ml/min and the IPA was delivered at 96 ml/min, thus producing a total volumetric flow rate of 100 ml/min.

25 mL of product was collected and this product was either retained as suspension or filtered over a 0.45 μm Durapore filter supplied by Millipore. Filtered samples were dried for 3.5 hours in a 30 °C incubator.

The continuous flow system was thoroughly cleaned and primed with pure solvent prior to coprecipitation.

Suspension samples were used to assess particle size using a Malvern Mastersizer fitted with a Small Volume Sample (SVS) system. Protein loading was measured using the BCA assay; activity was measured using the TAME assay.

Results:

Particle Sizing:

Sample	(Continuous) - Suspension
Particle Size (μm)	0.244

Measured Protein Loading:

Sample	(Continuous) - Filtered
Measured Protein Loading (%w/w)	3.31

Biological Activity

Sample	(Continuous) - Filtered
Activity by TAME assay (%)	54.3

Conclusion:

Viable PCMC can be made with a continuous precipitation process using the salt potassium sulfate as the coprecipitant and the coprecipitated protein remains biologically active.

Aim: Coprecipitation of pepsin, albumin or trypsin on potassium sulfate (K_2SO_4) PCMC from propan-2-ol (IPA) using a continuous flow coprecipitation systems – to provide suspension for zeta-potential measurements.

Experimental: Pepsin, trypsin (bovine pancreatic), albumin (bovine serum) and potassium sulfate Cat. No. 121K0043 and acetonitrile (MeCN) were supplied by Sigma Aldrich UK.

An aqueous solution containing 2.56 mg/ml protein (pepsin, albumin or trypsin) and 100 mg/ml potassium sulfate was prepared. This solution was precipitated into IPA saturated with potassium sulfate via the two line system, whereby the aqueous solution was delivered at 1 ml/min and the IPA was delivered at 99 ml/min, thus producing a total volumetric flow rate of 100 ml/min.

In both cases, 25 mL of product was collected and this product was either retained as suspension or filtered over a 0.45 μm Durapore filter supplied by Millipore. Filtered samples were dried for 3.5 hours in a 30 °C incubator.

The continuous flow system was thoroughly cleaned and primed with pure solvent prior to coprecipitation.

Suspension samples were used to assess particle size using a Malvern Zetasizer.

Results:

Zeta potential:

Sample	Zeta Potential (mV)
Pure, naked potassium sulfate	-20
Pepsin on potassium sulfate (JV845/1)	-46.1 ± 1.0
Albumin on potassium sulfate (JV845/2)	-25.6 ± 1.0
Trypsin on potassium sulfate (JV845/3)	-20.8 ± 0.8

Discussion:

PCMC can be efficiently formed by continuous coprecipitation of various proteins with the salt potassium sulphate. Pepsin is found to lower the zeta potential of an already negative crystal from -20 to ~-45 mV because the negative protein is concentrated on the surface. Albumin and trypsin, which are positive on the other hand, move the zeta potential in a positive direction. These data show the protein is bound and alters the zeta potential of the carrier crystal.

Conclusion:

Three proteins with very different isoelectric points alter the zeta potential of the PCMC particle. Hence the continuous process can be used to prepare viable PCMC, irrespective of protein type.